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(54) Title: THERMOSTABLE MUTANTS OF PYRROLOQUINOLINE QUINONE DEPENDENT GLUCOSE DEHYDROGENASE

(57) Abstract: The present invention relates to a mutant protein of PQQ-dependent s-GDH characterized in that in at least one of the positions 122 and 124 the amino acid lysine is present, wherein these positions correspond to the amino acid positions known from the *A. calcoaceticus* s-GDH wild-type sequence (SEQ ID NO: 2), it also discloses genes encoding such mutant s-GDH, and different applications of these sGDH mutants, particularly for determining the concentration of glucose in a sample.

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THERMOSTABLE MUTANTS OF PYRROLOQUINOLINE QUINONE DEPENDENT GLUCOSE DEHYDROGENASE

The present invention relates to a mutant protein of PQQ-dependent s-GDH characterized in that in at least one of the positions 122 and 124 the amino acid lysine is present, wherein these positions correspond to the amino acid positions known from the *A. calcoaceticus* s-GDH wild-type sequence (SEQ ID NO: 2), it also discloses genes encoding such mutant s-GDH, and different applications of these s-GDH mutants, particularly for determining the concentration of glucose in a sample.

Field of the invention:

The determination of blood glucose concentration is extremely important in clinical diagnosis and in the management of diabetes. Approximately 150 million people worldwide suffer from the chronic disease *diabetes mellitus*, a figure that may double by 2025 according to the WHO. Although diabetes is readily diagnosed and treated, successful long-term management requires low-cost diagnostic tools that rapidly and accurately report blood glucose concentrations. PQQ-dependent glucose dehydrogenases (EC 1.1.5.2) catalyze a reaction in which glucose is oxidized to gluconolactone. Consequently, this type of enzyme is used in measuring blood sugar. One of these tools is a diagnostic strip based on the soluble glucose dehydrogenase (s-GlucDOR, EC 1.1.5.2), a pyrroloquinoline quinone-containing enzyme originally derived from *Acinetobacter calcoaceticus.*

Quinoproteins use quinone as cofactor to oxidize alcohols, amines and aldoses to their corresponding lactones, aldehydes and aldolic acids (Duine, J.A., Energy generation and the glucose dehydrogenase pathway in *Acinetobacter* in "The Biology of *Acinetobacter*" (1991) 295-312, New York, Plenum Press; Duine, J.A., Eur. J. Biochem. 200 (1991) 271-284; Davidson, V.L., in "Principles and applications of quinoproteins" (1993), the whole book, New York, Marcel Dekker; Anthony, C., Biochem. J. 320 (1996) 697-711; Anthony, C. and Ghosh, M., Current Science 72 (1997) 716-727; Anthony, C., Biochem. Soc. Trans. 26 (1998) 413-417; Anthony, C. and Ghosh, M., Prog. Biophys. Mol. Biol. 69 (1998) 1-21. Among quinoproteins, those containing the noncovalently bound cofactor 2,7,9-tricarboxy-1H-pyrrolo [2,3-f]quinoline-4,5-dione (PQQ) constitute the largest sub-group (Duine 1991, supra). All bacterial quinone glucose dehydrogenases known so far belong to this

sub-group with PQQ as cofactor (Anthony and Ghosh 1997 supra; Goodwin, P.M. and Anthony, C., Adv. Microbiol. Physiol. 40 (1998) 1-80; Anthony, C., Adv. in Phot. and Resp. 15 (2004) 203-225).

Two types of PQQ-dependent glucose dehydrogenase (EC 1.1.5.2) have been characterized in bacteria: One is membrane-bound (m-GDH); the other is soluble (s-GDH). Both types do not share any significant sequence homology (Cleton-Jansen, A. M., et al., Mol. Gen. Genet. 217 (1989) 430-436; Cleton-Jansen, A. M., et al., Antonie Van Leeuwenhoek 56 (1989) 73-79; Oubrie, A., et al., Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 11787-11791). They are also different regarding both their kinetic as well as their immunological properties (Matsushita, K., et al., Bioscience Biotechnol. Biochem. 59 (1995) 1548-1555). The m-GDHs are widespread in Gram-negative bacteria, s-GDHs, however, have been found only in the periplasmatic space of *Acinetobacter* strains, like *A. calcoaceticus* (Duine, J.A., 1991a; Cleton-Jansen, A.M. et al., J. Bacteriol. 170 (1988) 2121-2125; Matsushita and Adachi, 1993) and *A. baumannii* (JP 11243949).

Through searching sequence databases, two sequences homologous to the full-length *A. calcoaceticus* s-GDH have been identified in *E.coli* K-12 and *Synechocystis sp.*. Additionally, two incomplete sequences homologous to *A. calcoaceticus* s-GDH were also found in the genome of *P. aeruginosa* and *Bordetella pertussis* (Oubrie et al. 1999 a, b, c) and *Enterobacter intermedium* (Kim, C.H. et al., Current Microbiol. 47 (2003) 457-461), respectively. The deduced amino acid sequences of these four uncharacterized proteins are closely related to *A. calcoaceticus* s-GDH with many residues in the putative active site absolutely conserved. These homologous proteins are likely to have a similar structure and to catalyze similar PQQ-dependent reactions (Oubrie et al., 1999 a, b, c; Oubrie A., Biochim. Biophys. Acta 1647 (2003) 143-151; Reddy, S., and Bruice, T.C., J. Am. Chem. Soc. 126 (2004) 2431-2438; Yamada, M. et al., Biochim. Biophys. Acta 1647 (2003) 185-192).

Bacterial s-GDHs and m-GDHs have been found to possess quite different sequences and different substrate specificity. For example, *A. calcoaceticus* contains two different PQQ-dependent glucose dehydrogenases, one designated m-GDH which is active *in vivo*, and the other designated s-GDH for which only *in vitro* activity can be shown. Cleton-Jansen et al., 1988; 1989 a, b cloned the genes coding for the two GDH enzymes and determined the DNA sequences of both of these GDH genes. There is no obvious homology between m-GDH and s-GDH

corroborating the fact that m-GDH and s-GDH represent two completely different molecules (Laurinavicius, V., et al, Biologija (2003) 31-34).

The gene of s-GDH from *A. calcoaceticus* has been cloned in *E. coli*. After being produced in the cell, the s-GDH is translocated through the cytoplasmic membrane into the periplasmic space (Duine, J.A., Energy generation and the glucose dehydrogenase pathway in *Acinetobacter* in "The Biology of *Acinetobacter*" (1991) 295-312, New York, Plenum Press; Matsushita, K. and Adachi, O., Bacterial quinoproteins glucose dehydrogenase and alcohol dehydrogenase in "Principles and applications of Quinoproteins" (1993) 47-63, New York, Marcel Dekker). Like the native s-GDH from *A. calcoaceticus*, recombinant s-GDH expressed in *E.coli* is a homodimer, with one PQQ molecule and three calcium ions per monomer (Dokter, P. et al., Biochem. J. 239 (1986) 163-167; Dokter, P. et al., FEMS Microbiol. Lett. 43 (1987) 195-200; Dokter, P. et al., Biochem. J. 254 (1988) 131-138; Olsthoorn, A. J. and Duine, J. A., Arch. Biochem. Biophys. 336 (1996) 42-48; Oubrie, A., et al., J. Mol. Biol. 289 (1999) 319-333; Oubrie, A., et al., Proc. Natl. Acad. Sci. U.S.A 96 (1999) 11787-11791; Oubrie, A., et al., Embo J. 18 (1999) 5187-5194). s-GDH oxidizes a wide range of mono- and disaccharides to the corresponding ketones which further hydrolyze to the aldonic acids, and it is also able to donate electrons to PMS (phenazine metosulfate), DCPIP (2,6-dichloro-phenolindophenol), WB (Wurster's blue) and short-chain ubiquinones such as ubiquinone Q1 and ubiquinone Q2 (Matsushita, K., et al., Biochem. 28 (1989) 6276-6280; Matsushita, K., et al., Antonie Van Leeuwenhoek 56 (1989) 63-72), several artificial electron acceptors such as N-methylphenazonium methyl sulfate (Olsthoorn, A. J. and Duine, J. A., Arch. Biochem. Biophys. 336 (1996) 42-48; Olsthoorn, A. J. and Duine, J. A., Biochem. 37 (1998) 13854-13861) and electro conducting polymers (Ye, L., et al., Anal. Chem. 65 (1993) 238-241). In view of s-GDH's high specific activity towards glucose (Olsthoorn, A. J. and Duine, J. A., (1996) supra) and its broad artificial electron acceptor specificity, the enzyme is well suited for analytical applications, particularly for being used in (bio-)sensor or test strips for glucose determination in diagnostic applications (Kaufmann, N. et al., Development and evaluation of a new system for determining glucose from fresh capillary blood and heparinized blood in "Glucotrend" (1997) 1-16, Boehringer Mannheim GmbH; Malinauskas, A.; et al., Sensors and Actuators, B: Chemical 100 (2004) 395-402).

Glucose oxidation can be catalyzed by at least three quite distinct groups of enzymes, i.e., by NAD/P-dependent glucose dehydrogenases, by flavoprotein glucose oxidases or by quinoprotein GDHs (Duine, J.A., Biosens. Bioelectronics 10 (1995) 17-23). A rather slow autooxidation of reduced s-GDH has been observed, demonstrating that oxygen is a very poor electron acceptor for s-GDH (Olsthoorn and Duine, 1996). s-GDH can efficiently donate electrons from the reduced quinone to mediators such as PMS, DCPIP, WB and short-chain ubiquinones such as Q1 and Q2, but it can not efficiently donate electrons directly to oxygen.

Traditional test strips and sensors for monitoring glucose level in blood, serum and urine e. g. from diabetic patients use glucose oxidase. The performance of the enzyme is dependent of the oxygen concentration. Glucose measurements at different altitudes with different oxygen concentrations in the air may lead to false results. The major advantage of PQQ-dependent glucose dehydrogenases is their independence from oxygen. This important feature is e.g., discussed in US 6,103,509, in which some features of membrane-bound GDH have been investigated.

An important contribution to the field has been the use of s-GDH together with appropriate mediators. Assay methods and test strip devices based on s-GDH are disclosed in detail in US 5,484,708. This patent also contains detailed information on the set-up of assays and the production of s-GDH-based test strips for measurement of glucose. The methods described there as well as in the cited documents are herewith included by reference.

Other patents or applications relating to the field and comprising specific information on various modes of applications for enzymes with glucose dehydrogenase activity are US 5,997,817; US 6,057,120; EP 0 620 283; and JP 11-243949-A.

A commercial system which utilizes s-GDH and an indicator that produces a color change when the reaction occurs (Kaufmann, et al., 1997, supra) is the Glucotrend® system distributed by Roche Diagnostics GmbH.

Despite the above discussed advantages for use of a PQQ dependent s-GDH, in the determination of glucose also a disadvantage has to be considered. The enzyme has rather a broad substrate spectrum as compared to m-GDH. That is, s-GDH oxidizes

not only glucose but also several other sugars including maltose, galactose, lactose, mannose, xylose and ribose (Dokter et al. 1986 a; Oubrie A., Biochim. Biophys. Acta 1647 (2003) 143-151). The reactivity towards sugars other than glucose may in certain cases impair the accuracy of determining blood glucose levels. In particular patients on peritoneal dialysis, treated with icodextrin (a glucose polymer) may contain in their body fluids, e.g., in blood, high levels of other sugars, especially of maltose (Wens, R., et al., Perit. Dial. Int. 18 (1998) 603-609).

Therefore clinical samples as e.g. obtained from diabetic patients, especially from patients with renal complications and especially from patients under dialysis may contain significant levels of other sugars, especially maltose. Glucose determinations in samples obtained from such critical patients may be impaired by maltose (Frampton, J. E., and Plosker, G. L., Drugs 63 (2003) 2079-2105).

There are few reports in the literature on attempts to produce modified PQQ-dependent s-GDHs with altered substrate specificity. Igarashi, S., et al., Biochem. Biophys. Res. Commun. 264 (1999) 820-824 report that introducing a point mutation at position Glu277 leads to mutants with altered substrate specificity profile.

Sode, EP 1 176 202, reports that certain amino acid substitutions within s-GDH lead to mutant s-GDH with an improved affinity for glucose. In EP 1 167 519 the same author reports on mutant s-GDH with improved stability. Furthermore the same author reports in JP2004173538 on other s-GDH mutants with improved affinity for glucose.

Kratzsch, P. et al., WO 02/34919 report that the specificity of s-GDH for glucose as compared to other sugar substrates, especially as compared to maltose, can be improved by amino acid substitutions in certain positions of s-GDH. Central and crucial is a substitution at amino acid position 348. A mutant s-GDH comprising for example a glycine in position 348 instead of a threonine as present in the wild-type s-GDH has a tremendously improved selectivity for the substrate glucose as, e.g. as compared to the substrate maltose.

However, whereas quite some improvements on glucose specificity have been reported, it appears that such improvements frequently go hand in hand with a reduced stability of s-GDH. For example, it has become evident that the improved

specificity of an s-GDH mutant comprising an amino acid substitution in position 348 goes to the expense of stability, especially to the expense of thermo stability. Stability, however, is crucial for example during production and for long term storage, e.g., of glucose test strips.

A great demand and clinical need therefore exists for mutant forms of s-GDH which feature a reasonable thermo stability or both a reasonable thermo stability and an improved specificity for glucose as a substrate.

It was the task of the present invention to provide new mutants or variants of s-GDH with significantly improved thermo stability as compared to the wild-type enzyme or as compared to a mutant with improved specificity but hampered stability.

It has been found that it is possible to significantly improve the thermo stability of wild-type s-GDH as well as of s-GDH mutants designed for improved specificity for glucose, e.g., of an s-GDH mutated at position 348, and to at least partially overcome the above described problems.

The thermo stability has been significantly improved by providing a mutant s-GDH according to the present invention as described in detail herein below and in the appending claims. Due to the improved thermo stability of the new forms of s-GDH, significant technical progress for glucose determinations in various fields of applications is possible.

The improved s-GDH mutants according to this invention can be used with great advantage for the specific detection or measurement of glucose in biological samples, especially by tests strip devices or by biosensors.

Summary of the invention:

The present invention relates to a mutant protein of PQQ-dependent s-GDH characterized in that in at least one of the positions 122 and 124 the amino acid lysine is present, and wherein these positions correspond to the amino acid positions known from the *A. calcoaceticus* s-GDH wild-type sequence (SEQ ID NO: 2).

Polynucleotide-sequences coding for a mutant protein of s-GDH according to the present invention as well as, an expression vector comprising such polynucleotide sequence, and a host cell comprising said expression vector also represent preferred embodiments of the invention.

The invention further relates to the use of a mutant according to the present invention in a method for measurement of glucose, especially by a tests strip device or with a biosensor.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1: Protein sequences of *A. calcoaceticus* PQQ-dependent s-GDH (top) and A. *baumannii* s-GDH (bottom) aligned according to sequence homology.

Figure 2: Illustration of pACSGDH vector referred to in Example 1 containing the wild-type or mutated DNA sequences, respectively, of soluble PQQ-dependent glucose dehydrogenase.

Figure 3: Nucleotide (DNA) sequence of the pACSGDH vector referred to in Example 1 containing the wild-type DNA sequence of soluble PQQ-dependent glucose dehydrogenase (SEQ ID NO: 16).

Detailed description of the invention:

In a first embodiment the invention relates to a mutant protein of PQQ-dependent s-GDH characterized in that in at least one of the positions 122 and 124 the amino acid lysine is present, wherein these positions correspond to the amino acid positions known from the *A. calcoaceticus* s-GDH wild-type sequence (SEQ ID NO: 2).

As discussed above, two completely different quinoprotein enzyme types with glucose dehydrogenase activity (membrane bound and soluble) are grouped together under EC 1.1.5.2. These two types appear not be related to each other.

For the purpose of this invention only the soluble form of GDH (s-GDH) is relevant and improved variants thereof are discussed herein below.

It is known in the art that the wild-type DNA-sequence of a soluble PQQ-dependent glucose dehydrogenase can be isolated from strains of *Acinetobacter*. Most preferred is the isolation from *Acinetobacter calcoaceticus*-type strain LMD 79.41. The polypeptide sequence of this wild-type s-GDH (the mature protein) is given in SEQ ID NO: 2 and the DNA sequence in SEQ ID NO: 1, respectively. Other LMD strains of *Acinetobacter* may also be used as source of wild-type s-GDH. Such sequences can be aligned to the sequence obtained from *A. calcoaceticus* and sequence comparisons be made. It also appears feasible to screen DNA-libraries of other bacterial strains, as for example described for *E.coli* K-12 (Oubrie, A., et al., J. Mol. Biol. 289 (1999) 319-333) and to identify sequences related to s-GDH in such genomes. Such sequences and yet unidentified homologous sequences may be used to generate s-GDH variants with improved thermo stability.

The achievements of the present invention are described in great detail by making reference to amino acid positions known from SEQ ID NO: 2, the wild-type sequence of s-GDH as isolated from *Acinetobacter calcoaceticus*-type strain LMD 79.41. Amino acid positions in different s-GDH isolates corresponding to positions of SEQ ID NO: 2 are easily identified by appropriate sequence comparison.

The multiple alignment and comparison of an s-GDH sequence with the wild-type sequence of SEQ ID NO: 2 preferably is performed with the PileUp program of GCG Package Version 10.2 (Genetics Computer Group, Inc.). PileUp creates a

multiple sequence alignment using a simplification of the progressive alignment method of Feng, D. F. and Doolittle, R. F., J. Mol. Evol. 25 (1987) 351-360, and the scoring matrixes for identical, similar, or different amino acid residues are defined accordingly. This process begins with the pair wise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster can then be aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences can be aligned by a simple extension of the pair wise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pair wise alignments that include increasingly dissimilar sequences and clusters, until all sequences have been included in the final pair wise alignment. This way amino acid positions in other, homologous s-GDH molecules be easily identified as corresponding to the positions given for *A. calcoaceticus* s-GDH in SEQ ID NO: 2. This is why the amino acid positions given herein shall be understood as amino acid positions of SEQ ID NO: 2 or as the positions corresponding thereto in another, homologous s-GDH molecule.

The term "mutant" or "variant" in the sense of the present invention relates to an s-GDH protein which compared to a corresponding wild-type sequence exhibits at least an amino acid substitution at a position corresponding to position 122 or 124 of SEQ ID NO: 2, wherein the amino acid present in the wild-type is substituted by lysine. The s-GDH mutant may comprise other substitutions and/or deletions and/or insertions provided that an s-GDH mutant of the invention does not differ by more than 50 amino acids from the s-GDH of SEQ ID NO: 2, e.g. that it exhibits at most 50 amino acid substitutions in total.

As mentioned above, improvements in glucose specificity appear to be possible largely at the expense of a reduced stability. As the skilled artisan will appreciate stability may relate to different aspects, like storage temperature and/or storage time, respectively. Short term temperature stress models are frequently used to assess stability. Stability according to the present invention is assessed in such a short term stress model and thus referred to as thermo stability. Thermo stability is determined by measuring the unstressed and stressed s-GDH enzyme activity of a sample. By setting the unstressed sample activity to 100% the remaining activity after stress treatment can be calculated in percent. For mutants of s-GDH with improved substrate specificity, 65°C for 30 minutes were chosen Using these conditions the wild-type enzyme has about 80 % of its original activity left, whereas

most of the mutants with improved specificity for glucose have only 10 % or less of their initial enzymatic activity left after performing this short-term stress model.

It has been found that two positions of s-GDH appear to be rather critical for achieving significant improvements in terms of thermo stability, i.e., positions 122 and 124. It has also been found that only the substitution by one of the 20 naturally occurring amino acids, the amino acid lysine, has a striking effect on thermo stability. The substitution by lysine at position 122 and/or 124 improves thermo stability of the wild-type s-GDH enzyme. The wild-type enzyme is rather stable at any rate but thermo stability is further improved by a lysine in position 122 and/or 124. What is of significant relevance is the fact that it has been found that these substitutions also have a pronounced effect on the thermo stability of mutants which previously had been generated in order to improve glucose specificity, but at the expense of a reduced thermo stability.

In a preferred embodiment the s-GDH mutant according to the present invention comprises a lysine in position 122 with said position corresponding to position 122 known from *A. calcoaceticus* wild-type s-GDH (SEQ ID NO: 2).

In another preferred embodiment the s-GDH mutant according to the present invention comprises a lysine in position 124 with said position corresponding to position 124 known *from A. calcoaceticus* wild-type s-GDH (SEQ ID NO: 2).

In still another preferred embodiment the s-GDH mutant according to the present invention comprises a lysine in position 122 and 124 with said positions corresponding to positions 122 and 124, respectively, known from *A. calcoaceticus* wild-type s-GDH (SEQ ID NO: 2).

As mentioned above, it is crucial that the thermo stability of s-GDH mutants with improved specificity for glucose, e.g., of the mutants disclosed in WO 02/34919 can be improved. It has been found and is demonstrated that this can be accomplished by the substitution of the naturally occurring amino acids by a lysine in position 122 and/or 124. In a very preferred embodiment the present invention therefore relates to an s-GDH mutant comprising a lysine in position 122 and/or 124 and additionally comprising one or more other modifications leading to an improved specificity for glucose, especially as compared to maltose.

A preferred mutant according to the present invention is characterized in that relative to the s-GDH wild-type enzyme as isolated from *A. calcoaceticus*, it has an at least two-fold improved substrate specificity for glucose as compared to at least one other selected sugar substrate and at the same time a thermo stability that is at least 20% of the thermo stability as measured for the wild-type enzyme.

In order to calculate the substrate specificity or cross-reactivity one easy way is to set the activity measured with glucose as substrate to 100 % and to compare the activity measured with the other selected sugar to the glucose value. Sometimes, in order not to be redundant, simply the term specificity is used without making special reference to glucose on the one hand and a selected other sugar substrate on the other hand.

The expert in the field will appreciate that comparison of enzymatic activities is best made at equimolar concentrations of the substrate molecules investigated using well-defined assay conditions. Otherwise corrections for differences in concentrations have to be made.

Standardized and well-defined assay conditions have to be chosen in order to assess (improvements in) substrate specificity. The enzymatic activity of s-GDH for glucose as a substrate as well as for other selected sugar substrates is measured as described in the Examples section.

Based on these measurements of enzymatic activity for glucose or a selected different sugar, preferably maltose, cross-reactivity (and improvements thereof) is assessed.

The s-GDH (cross-) reactivity for a selected sugar in percent is calculated as

Cross-reactivity [%] = (activity selected sugar/activity glucose) x 100%.

(Cross-) reactivity for maltose of wild-type s-GDH according to the above formula has been determined as about 105% (see Example 7).

(Improved) specificity is calculated according to the following formula:

$$\text{specificity (improvement)} = \frac{\text{activity glucose mutant}}{\text{activity selected sugar mutant}} \times \frac{\text{activity selected sugar wild-type}}{\text{activity glucose wild-type}}$$

As compared to the wild-type enzyme, an s-GDH form with an at least 10-fold improvement in specificity for glucose versus maltose (maltose / glucose) accordingly with maltose as substrate has at most 10.5 % of the activity as measured with glucose as substrate. Or, if, for example a mutant s-GDH has cross-reactivity for maltose of 20 % (determined and calculated as described above), this mutant as compared to the wild-type s-GDH therefore has a 5.25 fold improved substrate specificity (maltose / glucose).

The term "specific activity" for a substrate is well known in the art, it is preferably used to describe the enzymatic activity per amount of protein. Various methods are known to the art to determine specific activity of GDH molecules, using glucose or other sugars as substrates (Igarashi, S., et al., (1999) supra). A preferred method available for such measurement is described in detail in Example 8.

Whereas it is possible, to select many different sugar molecules and to investigate the glucose specificity of s-GDH in comparison to any such selected sugar molecule, it is preferred to select a clinically relevant sugar molecule for such a comparison. Preferred selected sugars are selected from the group consisting of mannose, allose, galactose, xylose, and maltose. Preferably, maltose or galactose is selected and mutant s-GDH is tested for improved substrate specificity for glucose as compared to galactose or maltose. In a further preferred embodiment the selected sugar is maltose.

In a preferred embodiment a mutant protein of PQQ-dependent s-GDH according to the present invention comprises a lysine at position 122 and/or 124 and additionally one or more amino acid substitution(s) at one or more position(s) selected from the group consisting of positions 16, 22, 65, 76, 116, 120, 127, 143, 168, 169, 171, 177, 224, 227, 230, 231, 245, 246, 255, 277, 287, 294, 295, 299, 302, 305, 307, 308, 317, 321, 323, 341, 348, 349, 354, 355, 364, 378, 422, 425, 428 and 438. Most of the above positions have been previously shown to influence the specificity of s-GDH for glucose as compared to other selected sugars, especially as

compared to the substrates maltose. The full disclosure of WO 02/34919 is herewith included by reference.

As described in WO 02/34919, a substitution of the amino acid in position 348 of the s-GDH sequence corresponding to the wild-type sequence isolated from *A. calcoaceticus,* can be used to significantly improve the glucose specificity of s-GDH. In an especially preferred embodiment the present invention relates to an s-GDH mutant comprising a lysine in position 122 and/or 124 and an amino acid substitution at position 348. Preferably the residue threonine at position 348 is substituted with an amino acid residue selected from the group consisting of alanine, glycine, and serine. In a more preferred embodiment glycine is used to substitute for threonine at position 348. The terminology T348G is known to the skilled artisan and indicates that threonine at position 348 is replaced by glycine.

In a further preferred embodiment the variant s-GDH comprising a lysine in position 122 and/or 124 is characterized in that the amino acid residue asparagine at position 428 is substituted with an amino acid residue selected from the group consisting of leucine, proline and valine. More preferred the substitution in position 428 is by proline.

One group of preferred s-GDH variants according to this invention comprising a lysine in position 122 and/or 124 is further characterized in that the amino acid residue threonine at position 348 and the amino acid asparagine at position 428 are both substituted, wherein preferred substitutions are the ones outlined above.

The mutants according to the present invention comprising a lysine in position 122 and/or 124 may optionally further be modified to comprise one or more amino acid substitutions at amino acid positions corresponding to positions 169, 171, 245, 341, and/or 349 of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 2).

In case the amino acid corresponding to position 169 of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 2) is substituted in a variant of the present invention, it is preferred that the naturally occurring amino acid leucine is substituted by phenylalanine, tyrosine or tryptophane. More preferred the substitution in position 169 is by phenylalanine.

In case the amino acid corresponding to position 171 of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 2) is substituted in a variant of the present invention, it is preferred that the naturally occurring amino acid tyrosine is substituted by an amino acid selected from the group consisting of from the group consisting of alanine, methionine, glycine. More preferred the substitution in position 171 is by glycine.

In case the amino acid corresponding to position 245 of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 2) is substituted in a variant of the present invention, it is preferred that the naturally occurring amino acid glutamic acid is substituted by aspartic acid, asparagine or glutamine. More preferred the substitution in position 245 is by aspartic acid.

In case the amino acid corresponding to position 341 of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 2) is substituted in a variant of the present invention, it is preferred that the naturally occurring amino acid methionine is substituted by valine, alanine, leucine or isoleucine. More preferred the substitution in position 341 is by valine.

In case the amino acid corresponding to position 349 of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 2) is substituted in a variant of the present invention, it is preferred that the naturally occurring amino acid valine is substituted by alanine or glycine. More preferred the substitution in position 349 is by alanine.

It has been also found that it is possible to further improve substrate specificity of an s-GDH variant by insertion of an amino acid between position 428 and 429. Such mutants comprising an amino acid insertion between amino acid 428 and amino acid 429 also represent a preferred starting material for generating mutants exhibiting both improved stability as well as improved specificity. In a preferred embodiment the present invention relates to a mutant s-GDH comprising a lysine in position 122 and/or 124 as well as an amino acid insertion between position 428 and position 429 of s-GDH.

In a further preferred embodiment the s-GDH mutant according to the present invention in addition to having a lysine in position 122 and/or 124 carries an insertion between amino acid residues 428 and 429 and comprises at least two

amino acid substitutions selected from the group consisting of positions 171, 245, 341, 348 and 349 as corresponding to amino acid positions of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 2).

In yet a further preferred embodiment the s-GDH mutant according to the present invention in addition to having a lysine in position 122 and/or 124 carries an insertion between amino acid residues 428 and 429 and comprises at least three amino acid substitutions selected from the group consisting of positions 171, 245, 341, 348 and 349 as corresponding to amino acid positions of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 2).

As the skilled artisan will appreciate, it is possible to undertake amino acid substitutions, e.g. silent mutations, which do not influence the properties of s-GDH to a significant extend. The variant according to the present invention will, however, have no more than 50 amino acid exchanges as compared to SEQ ID NO: 2. Preferably, the variant will comprise 20 or less amino acid substitutions, more preferred, only 10 amino acid substitutions or fewer substitutions will be present.

Some specific s-GDH variants according to the present invention are given in the Examples section. S-GDH variants with low glucose interference and acceptable thermo stability comprise mutants with substitutions in the following positions 65+122+124+171+245+341+348+426+428+430+436, 65+122+124+171+245+341+348+426+428+430 and 122+124+171+245+246+341+348+425+428, respectively. These three variants represent further preferred embodiments of the present invention.

Amino acid sequence analysis revealed that the sequence motives found in wild-type s-GDH from *A. calcoaceticus* on the one hand and *A. baumannii* on the other hand appear to be very conservative around the positions 122 and 124 that are of major relevance to improve thermo stability as identified and shown in the present invention.

A variant of PQQ-dependent s-GDH, comprising the amino acid sequence of TYNKSTD (SEQ ID NO: 3), wherein either the asparagine (N) or the serine (S) is replaced by a lysine represents a preferred embodiment of the present invention. SEQ ID NO: 3 corresponds to position 120-126 of *A. calcoaceticus* wild-type s-GDH or to position 120-126 of *A. baumannii* wild-type s-GDH but comprises either a

lysine in position 122 (=Xaa_1) or in position 124 (=Xaa_2) or in both these positions, thus replacing the naturally occurring amino acids asparagine or/and serine, respectively *(A. calcoaceticus)*.

Numerous possibilities are known in the art to produce mutant proteins. Based on the important findings of the present invention disclosing the critical importance of a lysine in position 122 and/or 124 of a mutant s-GDH the skilled artisan now can easily produce further appropriate variants of s-GDH harboring these and other favorable modifications. Such variants for example can be obtained by the methods known as random mutagenesis (Leung, D. W., et al., Technique 1 (1989) 11-15) and/or site directed mutagenesis (Hill, D. E., et al., Methods Enzymol. 155 (1987) 558-568). An alternative method to produce a protein with the desired properties is to provide chimaeric constructs, which contain sequence elements from at least two different sources or to completely synthesize an appropriate s-GDH gene. Such procedures known in the art may be used in combination with the information disclosed in the present invention to provide mutants or variants of s-GDH comprising e.g. additional amino acid substitutions in combination with the disclosed critical importance of a lysine in position 122 and/or 124, e.g., of SEQ ID NO: 2.

An s-GDH variant according to the present invention can e.g., be produced by starting from an s-GDH gene as isolated from *Acinetobacter calcoaceticus*-type strain LMD 79.41 as well as by starting from a homologous sequence. In the context of this application the term "homologous" is meant to comprise an s-GDH amino acid sequence with at least 90 % identity as compared to SEQ ID NO: 2. With other words, after appropriate alignment using the PileUp program, at least 90 % of the amino acids of such homologous s-GDH are identical to the amino acids described in SEQ ID NO: 2.

It will be understood that variations of DNA and amino acid sequences naturally exist, or may be intentionally introduced using methods known in the art. These variations may result in up to 10 % amino acid differences in the overall sequence, due to deletions, substitutions, insertions, inversions or additions of one or more amino acid residues in said sequence as compared to SEQ ID NO: 2. Such amino acid substitutions may be made, for example, on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic

acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine. Other contemplated variations include salts and esters of the afore mentioned polypeptides, as well as precursors of the aforementioned polypeptides, for example, precursors having an N-terminal substitution such as methionine, N-formylmethionine used as leader sequences. Such variations may be made without necessarily departing from the scope and the spirit of the present invention.

According to procedures known in the state of the art or according to the procedures given in the examples section, it is possible to obtain polynucleotide sequences coding for any of the s-GDH mutants as discussed above. The invention therefore comprises also isolated polynucleotide sequences encoding s-GDH mutant proteins according to the present invention as described above.

The present invention further includes an expression vector comprising a nucleic acid sequence according to the present invention operably linked a promoter sequence capable of directing its expression in a host cell.

The present invention further includes an expression vector comprising a nucleic acid sequence according to the present invention operably linked to a promoter sequence capable of directing its expression in a host cell. Preferred vectors are plasmids such as pACSGDH shown in Figures 2 and 3.

Expression vectors useful in the present invention typically contain an origin of replication, an antibiotic resistance for selection, a promoter for expression and the whole or part of the s-GDH gene variant. The expression vectors may also include other DNA sequences known in the art, like signal sequences (for a better folding, transportation into the periplasma or secretion), inducers for a better modulation of the expression, or cleavage sites for cloning.

The characteristics of the selected expression vector must be compatible to the host cell, which is to be employed. For example, when cloning in an *E.coli* cell system, the expression vector should contain promoters isolated from the genome of *E.coli* cells (e.g., *lac*, or *trp*). Suitable origins of replication like the ColE1 plasmid replication origin can be used. Suitable promoters include, for example, *lac* and *trp*.

It is also preferred that the expression vector includes a sequence coding for a selection marker like an antibiotic resistance gene. As selectable markers, ampicillin resistance, or canamycin resistance may be conveniently employed. All of these materials are known in the art and are commercially available.

Suitable expression vectors containing the desired coding and control sequences may be constructed using standard recombinant DNA techniques known in the art, many of which are described in Sambrook et al., in "Molecular Cloning: A Laboratory Manual" (1989) Cold Spring Harbor, NY, Cold Spring Harbour Laboratory Press.

The present invention additionally relates to host cells containing an expression vector which comprises a DNA sequence coding for all or part of the mutant s-GDH. The host cells preferably contain an expression vector that comprises all or part of one of the DNA sequences coding for a mutant s-GDH having one or more mutations shown in the Examples 2-8. Suitable host cells include, for example, *E.coli* HB101 (ATCC 33694) available from Promega (2800 Woods Hollow Road, Madison, WI, USA), XL1-Blue MRF' available from Stratagene (11011 North Torrey Pine Road, La Jolla, CA, USA) and the like.

Expression vectors may be introduced into host cells by various methods known in the art. For example, transformation of host cells with expression vectors can be carried out by polyethylene glycol mediated protoplast transformation method (Sambrook et al. 1989, supra). However, other methods for introducing expression vectors into host cells, for example, electroporation, bolistic injection, or protoplast fusion, can also be employed.

Once an expression vector containing an s-GDH variant has been introduced into an appropriate host cell, the host cell may be cultured under conditions permitting expression of the desired s-GDH variants. Host cells containing the desired expression vector with the DNA sequence coding for all or part of the mutant s-GDH can be easily identified by i.e. antibiotica selection. The expression of the s-GDH variants can be identified by different methods like measuring production of s-GDH mRNA transcripts, detection of the gene product immunologically or detection of the enzymatic activity of the gene product. Preferably an enzymatic assay is applied.

The present invention also teaches the generation and screening of s-GDH mutants. Random mutagenesis and saturation mutagenesis is performed as known in the art. Variants are screened for thermo stability (activity without heat stress treatment compared to remaining activity after heat stress treatment). The assay conditions chosen are adapted to ensure that the expected small enhancements brought about e.g., by a single amino acid substitution, can be measured. One preferred mode of selection or screening of appropriate mutants is given in Example 3. Any change or improvement as compared to the starting enzyme (mutant or wild-type) can be clearly detected.

It should, of course, be understood that not all expression vectors and DNA regulatory sequences would function equally well to express the DNA sequences of the present invention. Neither will all host cells function equally well with the same expression system. However, one of ordinary skill in the art will make an appropriate selection among the expression vectors, DNA regulatory sequences, and host cells using the guidance provided herein without undue experimentation.

The invention also relates to a process for producing s-GDH variants of the current invention comprising culturing a host cell of the invention under conditions suitable for production of the mutant s-GDH of the invention. For bacterial host cells, typical culture conditions are liquid medium containing carbon and nitrogen sources, the appropriate antibiotic and induction agent (depending on the used expression vector). Typical appropriate antibiotics include ampicillin, canamycin, chloroamphenicol, tetracycline and the like. Typical induction agents include IPTG, glucose, lactose and the like.

It is preferred that the polypeptides of the present invention are obtained by production in host cells expressing a DNA sequence coding the mutant s-GDH. The polypeptides of the present invention may also be obtained by *in vitro* translation of the mRNA encoded by a DNA sequence coding for the mutant s-GDH. For example, the DNA sequences may be synthesized as described above and inserted into a suitable expression vector, which in turn may be used in an *in vitro* transcription/translation system.

An expression vector comprising an isolated polynucleotide as defined and described above operably linked to a promoter sequence capable of promoting its

expression in a cell-free peptide synthesis system represents another preferred embodiment of the present invention.

The polypeptides produced e.g. by procedures as describe above, may then be isolated and purified using various routine protein purification techniques. For example, chromatographic procedures such as ion exchange chromatography, gel filtration chromatography and affinity chromatography may be employed.

One of the major applications of the improved s-GDH variants of this invention is for the use in test strips to monitor the blood-glucose level in diabetic patients. The insensitivity of PQQ-dependent glucose dehydrogenase towards oxygen is, as discussed above, a big advantage over glucose oxidase. The interference due to e.g., maltose, galactose, and/or other related sugars which may be present in a sample to be analyzed, can now be significantly reduced using the novel s-GDH variants having both improved thermo stability as well as improved specificity towards glucose. Of course many kinds of samples may be investigated. Bodily fluids like serum, plasma, intestinal fluid or urine are preferred sources for such samples.

The invention also comprises a method of detecting, determining or measuring glucose in a sample using an s-GDH mutant according to the present invention. It is especially preferred that the improved method for detection of glucose in a sample is characterized in that said detection, determination or measurement of glucose is performed using a sensor or test strip device.

Also within the scope of the present invention is a device for the detection or measurement of glucose in a sample comprising an s-GDH mutant according to this invention as well as other reagents required for said measurement.

The s-GDH variants with improved thermo stability of this invention can also be used to great advantage in biosensors (D'Costa, E. J., et al., Biosensors 2 (1986) 71-87; Laurinavicius, V., et al., Analytical Letters 32 (1999) 299-316; Laurinavicius, V., et al., Monatshefte fuer Chemie 130 (1999) 1269-1281; Malinauskas, A. et al., Sensors and Actuators, B: Chemical 100 (2004) 395-402) for online monitoring of glucose in a sample or a reactor. For this purpose, the s-GDH variants can, for example, be used to coat an oxygen-insensitive glassy electrode with an osmium complex containing a redox conductive epoxy network (Ye et al., 1993 supra) for more accurate determination of the glucose concentration.

In the following examples, all reagents, restriction enzymes, and other materials were obtained from Roche Diagnostics Germany, unless other commercial sources are specified, and used according to the instructions given by the suppliers. Operations and methods employed for the purification, characterization and cloning of DNA are well known in the art (Ausubel, F., et al., in "Current protocols in molecular biology" (1994), Wiley) and can be adapted as required by the skilled artisan.

The following examples further illustrate the present invention. These examples are not intended to limit the scope of the present invention, but provide further understanding of the invention.

Example 1

Cloning and expression of the wild-type *A. calcoaceticus* soluble PQQ-dependent glucose dehydrogenase in *E. coli*

The s-GDH gene was isolated from *Acinetobacter calcoaceticus* strain LMD 79.41 according to standard procedures. The wild-type s-GDH gene was subcloned into a plasmid containing the mgl promoter for adjustable expression (cf. Patent application WO 88/09373). The new construct was called pACSGDH (see Figures 2 and 3). The recombinant plasmids were introduced into a host organism selected from the *E.coli* group. These organisms were then cultivated under appropriate conditions and colonies showing s-GDH activity selected.

The plasmid pACSGDH was isolated from a 200 ml over-night culture of the clone mentioned above using the QIAGEN Plasmid Maxi Kit (Qiagen) according to the manufacturers' protocol. The plasmid was resuspended in 1 ml bi-distilled water. The concentration of the plasmid was determined using a Beckman DU 7400 Photometer.

The yield was 600 µg. Then the quality of the plasmid was determined by agarose gel electrophoresis.

Example 2:

Mutagenic PCR

To generate random mutations in the s-GDH-gene, mutagenic PCR (polymerase chain reaction) was performed. The pACSGDH plasmid and the DNA sequence encoding the mutated enzymes (PCR product from mutagenic PCR) were digested with the restriction enzymes *Sph* I and *Eco* RI. The products were gel purified. The digested DNA sequences were ligated and an aliquot of the ligation reaction mixture was used to transform competent *E.coli* cells. The transformants were subsequently selected on LB-plates containing ampicillin.

Individual colonies were chosen, grown over night in LB-medium containing ampicillin and subjected to screening (see Example 3).

Mutagenic PCR reaction mixture:
40 ng pACSGDH
1 x buffer without MgCl2 (Roche Diagnostics GmbH, Cat. 1699 105)
dCTP, dTTP 1mM
dATP, dGTP 0.2 mM (Roche Diagnostics GmbH, Cat. 1969 064)
40 pmol GF23-Primer (5′-CGC GCA CGC GCA TGC CGC CGA TGT TC) (= SEQ ID NO: 4)
40 pmol GR23 (5′-GAC GGC CAG TGA ATT CTT TTC TA) (= SEQ ID NO: 5)
7 mM MgCl2
0.6 mM MnCl2
5 U Taq DNA polymerase (Roche Diagnostics GmbH, Cat. 1146 165)
Gene Amp PCR System 2400 (Perkin Elmer), 30 cycles: 95 °C, 1 min, 45 °C 2 min, 72 °C 2 min

- Purification of the PCR products using the High Pure PCR Product Purification Kit from Roche Diagnostics GmbH (Cat. 1 732 676) according to the manufacturer's protocol.

- Digestion of the PCR-fragments with 25 U SphI (Roche Diagnostics GmbH, Cat. 606 120) in 1 x buffer H (Roche Diagnostics GmbH, Cat. 1 417 991) at 37 °C over night; addition of 25 U EcoRI (Roche Diagnostics GmbH, Cat. 703 737) and further digestion for 3.5 hours.

- Digestion of 50 µg pACSGDH with 180 U SphI and 180 U EcoRI in 1 x buffer H for 4 hours at 37 °C.

- Gel electrophoresis of the digested pACSGDH and the digested fragments using agarose gels (0.8 %).

- Extraction of the DNA molecules using QIAquick Gel Extraction Kit (Qiagen, Cat. 28706) according to the manufacturer's protocol.

- Determination of the concentration of the fragments and the digested vector using a Beckman DU 7400 Photometer.

- Determination of the quality of the purified products by agarose gel electrophoresis.

- Ligation of 100 ng digested vector with 140 ng mPCR-fragments using 1 U T4-DNA-Ligase (Roche Diagnostics GmbH, Cat. 481 220) in a volume of 20 µl at 16 °C over night.

- Electroporation of electrocompetent XL1F- cells (Stratagene) with 1 µl of the ligation reaction with 2.5 KV in 0.2 cm cuvettes using a BioRad *E.coli* Pulser (BioRad).

- After growth in 1 ml LB at 37 °C for one hour, bacteria were plated on LB-Ampicillin agar plates (100 µg / ml Ampicillin) and grown over night at 37 °C.

- 50 % of the expressed clones produced enzymatically active s-GDH that was subjected to the following screening method.

Example 3:

Screening for thermo stability

The mutant colonies on the agar plates described above where picked into microtiter plates (MTPs) containing 200 µl LB-Ampicillin-media/well and incubated over night at 37 °C. These plates are called master plates.

From each master plate, 5 µl sample/well was transferred to an MTP containing 5 µl per/well of B (B = Bacterial Protein Extraction Reagent; Pierce No. 78248) for cell disruption and 240 µl of 0.0556 mM pyrroloquinoline quinone (PQQ); 50 mM Hepes; 15 mM $CaCl_2$ pH 7.0/well for activation of s-GDH were added. To complete the formation of the holoenzyme, the MTP was incubated at 25 °C for 2 hours and at 10 °C over night. This plate is called working plate.

From the working plate 2 x 10 µl sample/cavity were transferred to two empty MTPs. One MTP was subjected to a short time temperature stress (30 minutes at 70°C /incubator). After cooling to room temperature the stressed and untreated MTP were tested with 90 µl of mediator solution containing 30 mM glucose (see Example 8).

The dE/min was calculated and the values from the unstressed samples were set to 100 % activity. The values obtained with the temperature stressed MTP were compared to the untreated values and calculated in percent activity ((e.g.: dE/min tempered/dE untreated)*100). This is equivalent to the thermo stability of the (variant) enzyme expressed in percent remaining activity. In order to compensate the deviations of results due to fluctuation of the distribution of heat in the MTPs during incubation, wild-type enzyme was added as reference to each plate in dedicated cavities.

The following mutant has been identified:

Enzyme	% remaining activity after 30minutes 70°C	Amino acid exchanges
WT	3-20 %	-
Mutant A	9-30 %	N122K, L202I

The variability of remaining activity is due to uneven heat distribution in the wells of the MTP and decay of holoenzyme during heating to apoenzyme and coenzyme and spontaneous reassembly to holoenzyme after thermal stress. Nevertheless the mutant A consistently showed a higher value for remaining enzymatic activity than the wild-type enzyme.

Example 4:

Sequencing of the gene coding for s-GDH mutant A

The plasmid containing the gene for s-GDH mutant A, which has a higher thermo stability than the wild-type was isolated (High Pure Plasmid Isolation Kit, Roche Diagnostics GmbH, No. 1754785) and sequenced using an ABI Prism Dye Terminator Sequencing Kit and ABI 3/73 and 3/77 sequencer (Amersham Pharmacia Biotech).

The following primers were used:

Sense strand:
GDH 1: 5′-TTA ACG TGC TGA ACA GCC GG-3′ (= SEQ ID NO: 6)
GDH 2: 5`-ATA TGG GTA AAG TAC TAC GC -3′ (= SEQ ID NO: 7)

Result:

The amino acid exchanges of mutant A already listed in the table of Example 3 were found.

Example 5:

s-GDH mutants obtained by saturation mutagenesis

Saturation mutagenesis was performed to see if both or only single amino acid exchanges in mutant A are responsible for the thermal stability improvement. Furthermore the method allows one to see if the found effect could be enhanced by other amino acid exchanges at the positions identified. The QuikChange Site-Directed Mutagenesis Kit (Stratagene, Cat. 200518) was used to substitute successively wild type amino acids at positions 122 and 202 of the wild-type s-GDH-protein, respectively.

The 5′- and the 3′-primer used for mutagenesis were complementary to each other and contained NNN in a central position. These 3 random synthesized nucleotides, which are at the desired position (122 or 202, respectively), were flanked by 12 to 16 nucleotides at each end which were identical to the sense and antisense DNA-strand of the template. Instead of the codon, the primer contained NNN therefore the oligonucleotides code for every possible codon.

For each of the positions 122 and 202, respectively, one PCR reaction was performed.

The PCR-reactions and the DpnI-restriction endonuclease digestions were performed according to the manual.

After that, 1 µl of each reaction was used for the electroporation of XL1F- cells. Cells were grown and the s-GDH-activities of the clones were determined as described above.

To increase the statistical likelihood that all 20 possible amino acids substitutions are covered in this evaluation, 200 clones were screened (see Example 3) for each position.

The following primers where used:

for position 122

Sense stand 5′- TCGTTATACCTATNNNAAATCAACAGATA -3′ (SEQ ID NO: 8)

Antisense strand 5′- TATCTGTTGATTTNNNATAGGTATAACGA-3′ (SEQ ID: NO: 9)

for position 202
Sense strand 5′-TAAAGTACTACGCNNNAATCTTGATGGAA-3′ (SEQ ID NO: 10)
Antisense strand 5′-TTCCATCAAGATTNNNGCGTAGTACTTTA -3′ (SEQ ID NO: 11)

Results:

The amino acid exchange at position 202 didn't change the thermo stability. Only the wobble at position 122 produced clones with enhanced thermal stability. The best exchange was N122K.

Example 6:

Generating mutants with high substrate specificity for glucose as compared to maltose and enhanced thermo stability

In WO 02/34919 several amino acid exchanges at different positions of s-GDH have been identified and shown to enhance the substrate specificity for glucose as compared to e.g., maltose. Combinations of the amino acid exchange T348G with amino acid substitutions at other positions for example at positions 169, 171, 245, 341 and/or 349 enhanced the substrate specificity furthermore. Several of these described mutants were selected to improve their thermo stability by introducing the found amino acid exchange N122K. The point mutation was accomplished by using the following primers.

Sense stand 5′- TCGTTATACCTATAAGAAATCAACAGATA -3′ (SEQ ID NO: 12)
Antisense strand 5′- TATCTGTTGATTTCTTATAGGTATAACGA-3′ (SEQ ID: NO: 13)

The same screening for thermo stability, as in Example 3 described, was applied, only the incubation temperature was reduced from 70 °C to 65 °C for 30 minutes.

Results:

Enzyme	Maltose/ Glucose (30 mM sugar in %)	% remaining activity after 30 minutes at 65 °C	Amino acid exchanges
WT	105 %	80 %	-
Template B/0	4 %	5 %	Y171G+E245D+M341V+ T348G+in429P
Mutant B/1	4 %	10 %	N122K+Y171G+E245D+M341V+ T348G+in429P
Template C/0	2 %	10 %	Y171G+E245D+M341V+ T348G+A426S+N428P+Q430M
Mutant C/1	2 %	20 %	N122K+Y171G+E245D+M341V+ T348G+A426S+N428P+Q430M
Template D/0	4 %	20 %	Y171G+E245D+Q246H+M341V+ T348G+T425V+N428P
Mutant D/1	4 %	30 %	Y171G+N122K+E245D+Q246H+M341V+ T348G+T425V+N428P

It can be clearly seen that on all mutant types the additional amino acid exchange N122K produced an enhancement of thermo stability in the chosen stress model without affecting the substrate specificity.

Example 7:

Generating mutants with high substrate specificity and further enhanced thermo stability

While screening for enhanced substrate specificity as e. g. described in WO 02/34919, using m-PCR and saturation mutagenesis at random positions the spectrum of screening parameters was expanded for thermo stability as above described.

Starting with a mutant E/0 with high substrate specificity for glucose compared to maltose (2%) with the amino acid exchanges Y171G+E245D+M341V+ T348G+N428P a new amino acid exchange S124K was found.

This new exchange was then applied to the already improved mutants containing N122K of Example 6 using the following primers:

Sense strand 5′- CCTATAAGAAAAAGACAGATACGCTCG -3′ (SEQ ID NO: 14)

Antisense strand 5′- CGAGCGTATCTGTCTTTTTCTTATAGG-3′ (SEQ ID: NO: 15)

Results:

Enzyme	Maltose/Glucose (30 mM sugar in %)	% remaining activity after 30 minutes at 65 °C	Amino acid exchanges
WT	105 %	80 %	-
Mutant B/1	4 %	10 %	N122K+Y171G+E245D+M341V+ T348G+in429P
Mutant B/2	4 %	15 %	N122K+S124K+Y171G+E245D+M341 V+ T348G+in429P
Mutant C/1	2 %	20 %	N122K+Y171G+E245D+M341V+ T348G+A426S+N428P+Q430M
Mutant C/2	2 %	25 %	N122K+S124K+Y171G+E245D+M341 V+ T348G+A426S+N428P+Q430M
Mutant D/1	4 %	30 %	N122K+Y171G+E245D+Q246H+M341 V+ T348G+T425V+N428P
Mutant D/2	4 %	40 %	N122K+124K+Y171G+E245D+Q246H +M341V+ T348G+T425V+N428P
Mutant E/0	2 %	10 %	Y171G+E245D+M341V+ T348G+N428P
Mutant E/1	2%	20%	Y171G+S124K+E245D+M341V+T348 G+N428P

The above results show that the amino acid exchanges on positions N122K and S124K have an additive, positive effect on the thermo stability of the mutants.

Example 8:

Purification of wild-type or variant s-GDH and analysis of enzymatic activity respectively

E. coli cells were grown (LB-Amp. 37 °C), harvested and resuspended in potassium phosphate buffer pH 7.0. Cell disruption was performed by French Press passage (700-900 bar). After centrifugation the supernatant was applied to an S-Sepharose (Amersham Pharmacia Biotec) column equilibrated with 10 mM potassium phosphate buffer pH 7.0. After washing, the s-GDH was eluted using a salt gradient 0-1 M NaCl. The fractions showing s-GDH activity were pooled, dialyzed against potassium phosphate buffer pH 7.0 and re-chromatographed on re-equilibrated S-Sepharose column. The active fractions were pooled and subjected to a gel filtration using a Superdex® 200 column (Amersham). The active fractions were pooled and after addition of $CaCl_2$ (3 mM end concentration) stored at –20 °C.

Enzyme assay and protein determination of purified wild-type and variant s-GDH, respectively

Protein determination was performed using the Protein Assay Reagent no. 23225 from Pierce (calibration curve with BSA, 30 Min. 37 °C).

The s-GDH samples were diluted to 1 mg protein/ml with 0.0556 mM pyrollo-quinoline quinone(PQQ); 50 mM Hepes; 15 mM $CaCl_2$ pH 7.0 and incubated at 25 °C for 30 minutes for reconstitution or activation.

After activation, samples were diluted with 50 mM Hepes; 15 mM $CaCl_2$ pH 7.0 to approximately 0,02 U/ml, and 50 µl of each diluted sample was added to 1000 µl of a 0.2 M citrate buffer solution (pH 5.8; at 25 °C) containing 0.315 mg (4-(dimethylphosphinylmethyl)-2-methyl-pyrazolo-[1.5a]-imidazol-3-yl)-(4-nitro-sophenyl)-amine (see patent US 5,484,708)/ml as a mediator and 30 mM sugar).

Extinction at 620 nm is monitored during the first 5 minutes at 25 °C.

One Unit enzyme activity corresponds the conversion of 1 mMol mediator/min under the above assay conditions

Calculation: Activity = (total volume * dE/min [U/ml]) : (ε * sample volume * 1)

(ε = coefficient of extinction; $\varepsilon_{620\,nm} = 30[1^* mmol^{-1} * cm^{-1}]$).

The assay was performed with glucose and maltose (Merck, Germany), respectively.

Results:

Enzyme	M/G (30 mM sugar in %)	U/mg Protein.	Amino acid exchanges
WT	105 %	800	-
B/2	4 %	106	N122K+S124K+Y171G+E245D+M341V+ T348G+in429P
C/0	2 %	435	Y171G+E245D+M341V+ T348G+A426S+N428P+Q430M
C/2	2 %	450	N122K+S124K+Y171G+E245D+M341V+ T348G+A426S+N428P+Q430M
D/2	4 %	441	N122K+S124K+Y171G+E245D+Q246H+M 341V+ T348G+T425V+N428P

Example 9:

Comparative temperature stability of purified mutants with and without amino acid exchanges N122K and S124K

The purified s-GDH samples of wild-type, mutant C/0 and C/2 (Example 8) were subjected to an alternative temperature stress model which resembles production and/or transport temperature stress conditions. Solutions of 1 mg enzyme protein/20 mM potassium phosphate pH 7.0; 0.016 mg PQQ/ml were made to activate the s-GDHs. After incubation for 30 minutes at room temperature the initial activity towards glucose was determined (see Example 8) and the samples incubated in the waterbath at 48 °C. After 30 minutes of temperature stress the remaining activity was measured and calculated in percent (in comparison to the initial activity).

Results:

Enzyme	Remaining activity
Wilde-type	99 %
C/0	66 %
C/2	94 %

The impact of the amino acid exchanges on positions N122K and S124K and the resulting improvement of temperature stability can be clearly seen, also under these alternative temperature stress conditions.

Example 10:

Determination of glucose in the presence or absence of maltose

The wild-type s-GDH and variants B/2, C/2 and D/2 of s-GDH, respectively, can be applied for glucose determination in the presence or absence of maltose. The reference sample contains 50 mg glucose/dl. The "test"-samples contain 50 mg glucose/dl and 100 or 200 mg/dl maltose, respectively. Enzyme solutions with the same amounts of s-GDH activity (for example 5 U/ml; activity as determined in Example 8) are used for each assay.

In a cuvette are mixed:

1 ml 0.315 mg (4-(dimethylphosphinylmethyl)-2-methyl-pyrazolo-[1.5a]-imidazol-3-yl)-(4-nitrosophenyl)-amine ml/0.2 M citrate pH 5.8

0.033 ml reference or test sample

The assay is started by adding 0.050 ml of the s-GDH enzyme solution (which is an excess of s-GDH for conversion of glucose) to the cuvette. The change of absorption at 620 nm is monitored. After 2-5 minutes constant values are observed and the dE/5 min is calculated. The value obtained by measuring the reference sample with wild-type s-GDH is set to 100%. The other values are compared to this reference value and calculated in %.

Clearly less maltose interference is detected in the test samples when using the novel also more stable variants of s-GDH according to this invention.

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Patent Claims

1. A mutant protein of PQQ-dependent s-GDH characterized in that in at least one of the positions 122 and 124 the amino acid lysine is present, wherein these positions correspond to the amino acid positions known from the *A. calcoaceticus* s-GDH wild-type sequence (SEQ ID NO: 2).

2. The mutant of claim 1, wherein the amino acid at position 122 is a lysine.

3. The mutant of claim 1, wherein the amino acid at position 124 is a lysine.

4. The mutant of claim 1, wherein the amino acid lysine is present at both the positions 122 and 124.

5. The mutant according to any of claims 1 to 4, additionally comprising (an) amino acid substitution(s) at one or more position(s) selected from the group consisting of positions 16, 22, 65, 76, 116, 120, 127, 143, 168, 169, 171, 177, 224, 227, 230, 231, 245, 246, 255, 277, 287, 294, 295, 299, 302, 305, 307, 308, 317, 321, 323, 341, 348, 349, 354, 355, 364, 378, 422, 425, 428 and 438.

6. The mutant according to claim 5, wherein said one or more additional amino acid substitution(s) is a substitution at position 348.

7. The mutant according to claim 5, wherein said one or more additional amino acid substitution(s) is a substitution at position 428.

8. The mutant according to claim 5, wherein said one or more additional amino acid substitution(s) is a substitution at both the positions 348 and 428.

9. The mutant of claim 6, wherein threonine at position 348 is replaced by alanine, glycine, or serine.

10. An isolated polynucleotide encoding the s-GDH mutant protein according to any of claims 1 to 9.

11. An expression vector comprising an isolated polynucleotide as defined in claim 10 operably linked to a promoter sequence capable of promoting the expression of said polynucleotide in a host cell.

12. A host cell comprising the expression vector of claim 11.

13. A process for producing s-GDH variants comprising culturing the host cell of claim 12 under conditions suitable for production of the enzyme variants.

14. A method of detecting, determining or measuring glucose in a sample using an s-GDH mutant according to any of claims 1 to 9, said improvement comprising contacting the sample with the mutant.

15. The method of claim 14 further characterized in that said detection, determination or measurement of glucose is performed using a sensor or test strip device.

16. A device for the detection or measurement of glucose in a sample comprising an s-GDH mutant according to any of claims 1 to 9 and other reagents required for said measurement.

Fig. 1

Amino acid sequences of A. calcoaceticus (top) and
A. baumannii (bottom)

```
                .         .         .         .         .
      1 DVPLTPSQFAKAKSENFDKKVILSNLNKPHALLWGPDNQIWLTERATGKI 50
        |:|||||.||||||.|||||||||||||||||||||||||||||||||||
      1 DIPLTPAQFAKAKTENFDKKVILSNLNKPHALLWGPDNQIWLTERATGKI 50
                .         .         .         .         .
     51 LRVNPESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTF 100
        ||||| ||| ||||||||||.||||||||||||||||||.||||||||||
     51 LRVNPVSGSAKTVFQVPEIVSDADGQNGLLGFAFHPDFKHNPYIYISGTF 100
                .         .         .         .         .
    101 KNPKSTDKELPNQTIIRRYTYNKSTDTLEKPVDLLAGLPSSKDHQSGRLV 150
        ||||||||||||||||||||||||.||| |||:||:||||||||||||||
    101 KNPKSTDKELPNQTIIRRYTYNKTTDTFEKPIDLIAGLPSSKDHQSGRLV 150
                .         .         .         .         .
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                .         .         .         .         .
    201 RLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPNS 250
        |||||||||||||||||||||||||||||||||||| ||||||||||||
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                .         .         .         .         .
    251 DDEINLIVKGGNYGWPNVAGYKDDSGYAYANYSAAANKS.IKDLAQNGVK 299
        ||||||:.|||||||||||||||||||||||||||| ||| ||||||||:|
    251 DDEINLVLKGGNYGWPNVAGYKDDSGYAYANYSAATNKSQIKDLAQNGIK 300
                .         .         .         .         .
    300 VAAGVPVTKESEWTGKNFVPPLKTLYTVQDTYNYNDPTCGEMTYICWPTV 349
        || |||||||||||||||||||||||||||||||||||| |||||||
    301 VATGVPVTKESEWTGKNFVPPLKTLYTVQDTYNYNDPTCGEMAYICWPTV 350
                .         .         .         .         .
    350 APSSAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVP 399
        |||||||| |||||| ||||||||||||||||||||||||||| |||:|
    351 APSSAYVYTGGKKAIPGWENTLLVPSLKRGVIFRIKLDPTYSTTLDDAIP 400
                .         .         .         .         .
    400 MFKSNNRYRDVIASPDGNVLYVLTDTAGNVQKDDGSVTNTLENPGSLIKF 449
        ||||||||||||||:|| ||||||||||||||||||.||||||||||
    401 MFKSNNRYRDVIASPEGNTLYVLTDTAGNVQKDDGSVTHTLENPGSLIKF 450

    450 TYKAK 454
        ||  |
    451 TYNGK 455
```

Fig. 2

Schematic diagram of the plasmide with gene for s-GDH (pACSGDH)

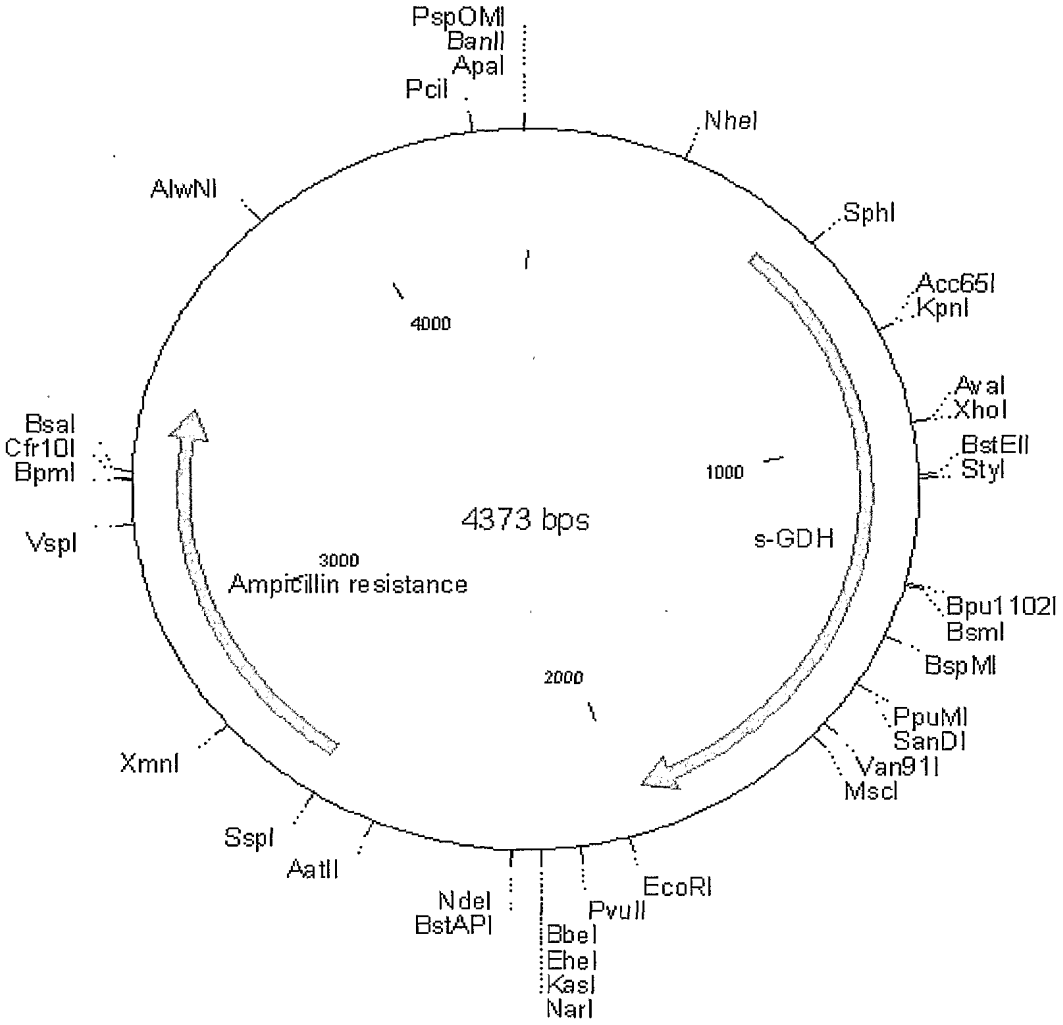

Fig. 3a

Sequence vector pACSGDH

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Fig. 3b

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1               5                   10                  15

ttt gac aag aaa gtt att cta tct aat cta aat aag ccg cac gcg ttg       96
Phe Asp Lys Lys Val Ile Leu Ser Asn Leu Asn Lys Pro His Ala Leu
            20                  25                  30

tta tgg gga cca gat aat caa att tgg tta act gag cga gca aca ggt      144
Leu Trp Gly Pro Asp Asn Gln Ile Trp Leu Thr Glu Arg Ala Thr Gly
        35                  40                  45

aag att cta aga gtt aat cca gag tcg ggt agt gta aaa aca gtt ttt      192
Lys Ile Leu Arg Val Asn Pro Glu Ser Gly Ser Val Lys Thr Val Phe
    50                  55                  60

cag gta cca gag att gtc aat gat gct gat ggg cag aat ggt tta tta      240
Gln Val Pro Glu Ile Val Asn Asp Ala Asp Gly Gln Asn Gly Leu Leu
65                  70                  75                  80

ggt ttt gcc ttc cat cct gat ttt aaa aat aat cct tat atc tat att      288
Gly Phe Ala Phe His Pro Asp Phe Lys Asn Asn Pro Tyr Ile Tyr Ile
                85                  90                  95

tca ggt aca ttt aaa aat ccg aaa tct aca gat aaa gaa tta ccg aac      336
Ser Gly Thr Phe Lys Asn Pro Lys Ser Thr Asp Lys Glu Leu Pro Asn
            100                 105                 110
```

```
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Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ser Thr Asp Thr Leu
        115                 120                 125

gag aag cca gtc gat tta tta gca gga tta cct tca tca aaa gac cat      432
Glu Lys Pro Val Asp Leu Leu Ala Gly Leu Pro Ser Ser Lys Asp His
    130                 135                 140

cag tca ggt cgt ctt gtc att ggg cca gat caa aag att tat tat acg      480
Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Gln Lys Ile Tyr Tyr Thr
145                 150                 155                 160

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Ile Gly Asp Gln Gly Arg Asn Gln Leu Ala Tyr Leu Phe Leu Pro Asn
                165                 170                 175

caa gca caa cat acg cca act caa caa gaa ctg aat ggt aaa gac tat      576
Gln Ala Gln His Thr Pro Thr Gln Gln Glu Leu Asn Gly Lys Asp Tyr
            180                 185                 190

cac acc tat atg ggt aaa gta cta cgc tta aat ctt gat gga agt att      624
His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile
        195                 200                 205

cca aag gat aat cca agt ttt aac ggg gtg gtt agc cat att tat aca      672
Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Val Ser His Ile Tyr Thr
    210                 215                 220

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225                 230                 235                 240

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            260                 265                 270

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Asp Asp Ser Gly Tyr Ala Tyr Ala Asn Tyr Ser Ala Ala Ala Asn Lys
        275                 280                 285

tca att aag gat tta gct caa aat gga gta aaa gta gcc gca ggg gtc      912
Ser Ile Lys Asp Leu Ala Gln Asn Gly Val Lys Val Ala Ala Gly Val
    290                 295                 300

cct gtg acg aaa gaa tct gaa tgg act ggt aaa aac ttt gtc cca cca      960
Pro Val Thr Lys Glu Ser Glu Trp Thr Gly Lys Asn Phe Val Pro Pro
305                 310                 315                 320

tta aaa act tta tat acc gtt caa gat acc tac aac tat aac gat cca     1008
Leu Lys Thr Leu Tyr Thr Val Gln Asp Thr Tyr Asn Tyr Asn Asp Pro
                325                 330                 335
```

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Thr Cys Gly Glu Met Thr Tyr Ile Cys Trp Pro Thr Val Ala Pro Ser
            340                 345                 350

tct gcc tat gtc tat aag ggc ggt aaa aaa gca att act ggt tgg gaa      1104
Ser Ala Tyr Val Tyr Lys Gly Gly Lys Lys Ala Ile Thr Gly Trp Glu
        355                 360                 365

aat aca tta ttg gtt cca tct tta aaa cgt ggt gtc att ttc cgt att      1152
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Lys Leu Asp Pro Thr Tyr Ser Thr Thr Tyr Asp Asp Ala Val Pro Met
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Phe Lys Ser Asn Asn Arg Tyr Arg Asp Val Ile Ala Ser Pro Asp Gly
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Lys Ile Leu Arg Val Asn Pro Glu Ser Gly Ser Val Lys Thr Val Phe
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Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Gln Lys Ile Tyr Tyr Thr
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Ile Gly Asp Gln Gly Arg Asn Gln Leu Ala Tyr Leu Phe Leu Pro Asn
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His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile
195 200 205

Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Val Ser His Ile Tyr Thr
210 215 220

Leu Gly His Arg Asn Pro Gln Gly Leu Ala Phe Thr Pro Asn Gly Lys
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Leu Leu Gln Ser Glu Gln Gly Pro Asn Ser Asp Asp Glu Ile Asn Leu
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260 265 270

Asp Asp Ser Gly Tyr Ala Tyr Ala Asn Tyr Ser Ala Ala Ala Asn Lys
275 280 285

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Pro Val Thr Lys Glu Ser Glu Trp Thr Gly Lys Asn Phe Val Pro Pro
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Leu Lys Thr Leu Tyr Thr Val Gln Asp Thr Tyr Asn Tyr Asn Asp Pro
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Thr Cys Gly Glu Met Thr Tyr Ile Cys Trp Pro Thr Val Ala Pro Ser
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Ser Ala Tyr Val Tyr Lys Gly Gly Lys Lys Ala Ile Thr Gly Trp Glu
        355                 360                 365

Asn Thr Leu Leu Val Pro Ser Leu Lys Arg Gly Val Ile Phe Arg Ile
    370                 375                 380

Lys Leu Asp Pro Thr Tyr Ser Thr Thr Tyr Asp Asp Ala Val Pro Met
385                 390                 395                 400

Phe Lys Ser Asn Asn Arg Tyr Arg Asp Val Ile Ala Ser Pro Asp Gly
                405                 410                 415

Asn Val Leu Tyr Val Leu Thr Asp Thr Ala Gly Asn Val Gln Lys Asp
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/011077

A. CLASSIFICATION OF SUBJECT MATTER
C12N9/04 C12N15/53 C12Q1/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, Sequence Search, WPI Data, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OUBRIE A: "Structure and mechanism of soluble glucose dehydrogenase and other PQQ-dependent enzymes" BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - PROTEINS & PROTEOMICS, ELSEVIER, vol. 1647, no. 1-2, 11 April 2003 (2003-04-11), pages 143-151, XP004417978 ISSN: 1570-9639 cited in the application abstract page 148	1,2, 10-12
A	EP 1 367 120 A (TOYO BOSEKI) 3 December 2003 (2003-12-03) claims 1-30; tables 2,6,9,14	1-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 January 2006	24/01/2006

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/011077

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 1 176 202 A (SODE KOJI) 30 January 2002 (2002-01-30) cited in the application	1-16
A	DATABASE GENESEQ 'Online! 9 September 2004 (2004-09-09), "Modified pyrrolo quinoline quinone dependant glucose dehydrogenase" XP002308323 Database accession no. ADP90774 the whole document	1-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2005/011077

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] in written format

   [X] in computer readable form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [X] filed together with the international application in computer readable form

   [ ] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/011077

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1367120 A	03-12-2003	US 2003232418 A1	18-12-2003
EP 1176202 A	30-01-2002	CA 2372741 A1	09-11-2000
		CN 1353759 A	12-06-2002
		WO 0066744 A1	09-11-2000
		TW 224136 B	21-11-2004